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(54) Title: XANTHONE ANALOGS FOR THE TREATMENT OF INFECTIOUS DISEASES

(57) Abstract

    Therapeutic compositions for the treatment of infectious diseases are disclosed. These compositions comprise xanthones and xanthone derivatives, such as 2,3,4,5,6-pentahydroxyxanthone. Also disclosed are methods for the treatment of infectious diseases using such compounds.

XANTHONE ANALOGS FOR THE TREATMENT
OF INFECTIOUS DISEASES

TECHNICAL FIELD

5   This invention pertains to therapeutic
compositions for the treatment of infectious diseases.

15   ### BACKGROUND OF THE INVENTION
        Protozoan parasites cause diseases such as
malaria, trypanosomiasis, Chagas' disease,
leishmaniasis, giardiasis, and amoebiasis. These and
other protozoan parasite diseases have historically
20   occurred in tropical and sub-tropical areas where they
cause widespread damage to human populations. Although
they receive little attention in the Western world,
protozoan diseases affect more people worldwide than
diseases brought on by any other biological cause
25   (Heyneman, 1988).
        Today, malaria remains the most destructive
single infectious disease in the developing world. It
is responsible for more human energy loss, more
debilitation, more loss of work capacity, and more
30   economic damage than any other human ailment facing the
world today (Heyneman, 1988). The World Health
Organization estimates that 1 to 2 million deaths are
caused by malaria each year in Africa alone; most of
these are children under the age of five (World Health
35   Organization, 1991). In addition, over 300 million
people worldwide are believed to be chronically
infected, and each year nearly one third of these

individuals will suffer acute manifestations of the disease.

Today, the pathologic capacity of protozoa is being increasingly demonstrated in the Western world among the victims of AIDS (Acquired Immunodeficiency Syndrome). AIDS depletes the immune system of affected individuals; this allows opportunistic agents which would be defeated by an active immune system to infect AIDS patients. Several protozoa have emerged as important opportunistic infections in AIDS patients including *Cryptosporidium parvum*, *Entamoeba histolytica*, *Giardia lamblia*, *Pneumocystis carinii* (which may be a fungal or protozoal pathogen), and *Toxoplasmosis gondii*.

Despite the prevalence and significance of protozoan infections, therapy for these diseases is generally poor or in need of improvement. Many chemotherapeutic agents used to treat protozoan infections are non-specific cytotoxins that are highly toxic and cause severe side effects in patients. However, these drugs are used because there are no better alternatives. For example, giardiasis and amoebiasis are treated using metronidazole (a nitroimidazole), but the use of this drug is clouded by its mutagenic potential (Campbell, 1986) and its adverse interaction with alcohol. For trypanosomiasis and leishmaniasis standard therapies (suramin, melarsoprol, and pentavalent antimonials) are dangerously toxic, occasionally fatal, and often ineffective (Mebrahtu, 1989; Grogl et al., 1992). Other drugs are becoming ineffective due to emerging resistance. In the case of malaria, effective therapy has previously been provided by chloroquine but its efficacy is now threatened by the rapid emergence of drug resistant strains of *Plasmodium falciparum*, the causative agent for the most severe, often fatal, form of the disease (Cowman, 1990). Other protozoal infections such as cryptosporidiosis or Chagas' disease have no proven curative agent.

The present invention is directed to new antiparasitic agents and methods of using known compounds as anti-parasitic agents. The invention is also directed to compounds with broad-spectrum anti-
5   microbial activity.

SUMMARY OF THE INVENTION

As a result of studies aimed at developing new anti-parasitic agents, the present inventors have
10   discovered that xanthones and a wide range of xanthone derivatives and structurally related compounds, as shown in formula X1 below, have potent anti-parasitic activity. The compounds have broad-spectrum anti-microbial activity, including anti-fungal activity
15   against *Candida albicans* and *Apergillus fumigatus*.

Formula X1:

20

wherein:
25   A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, aryl or haloaryl; and
R$_1$-R$_8$ are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl,
30   alkene, substituted alkyl (such as alkylamine, alkylthio and haloalkyl), amino, ester, ether and nitro groups and O-linked and C-linked carbohydrates.
Examples of substituted antimony include antimonial oxides and antimony substituted with hydroxy,
35   chlorine, alkyl and aryl groups (e.g. SbCl, SbCl$_3$, SbOH, Sb(O)(OH)).
In another embodiment, A is oxygen, sulfur or NH, and R$_1$-R$_8$ are independently selected from the group

consisting of H, OH, aryl, haloaryl, arylamine, alkyl, alkene substituted alkyl, halogen, amino, ester, ether and nitro groups.
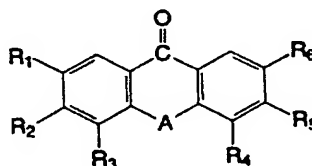
In other embodiments, A is oxygen and R_1-R_8 are
5   independently selected from the group consisting of H, OH, and acetoxy ($CH_3C(O)O$).

The formula X1 compounds encompass some compounds described for the first time herein.

In other embodiments, the compounds of the
10   present invention include compounds according to formula X2:

Formula X2:

15

wherein A is oxygen, sulfur or NH, and R_1-R_6 are
20   independently selected from the group consisting of H, OH, aryl, arylamine, alkyl, alkene substituted alkyl, halogen, amino, ester, ether and nitro groups. In particular embodiments of the present invention, the term "alkyl" refers to substituents having lower alkyl
25   groups, i.e., C_1-C_{10}.

In preferred embodiments of the present invention, the formula X1 compounds are compounds according to the formulae:

30

Formula X3:

35
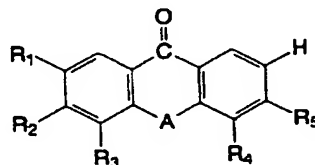
Formula X4:

5

wherein A is selected from the group consisting of
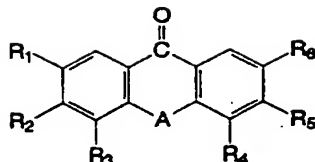oxygen, sulfur and NH, and R_1-R_6 are independently
10   selected from the group consisting of H, OH or an ester
group, such as $OCOCH_3$ or $OCO(CH_2)_3CH_3$ or a carbamate
ester. In preferred embodiments, the R_2 and R_5 groups
are esters. In yet other embodiments, A is oxygen and
R_2-R_5 are all hydroxy; in further preferred embodiments,
15   at least one of R_1 and R_6 are H.

In more preferred embodiments, the formula X1
compounds are compounds having the following formulae:
Formula X5:

20
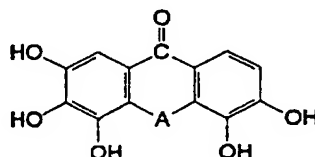
25   Formula X5E:

30

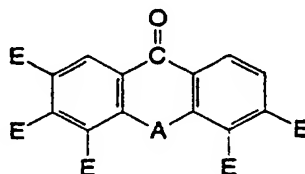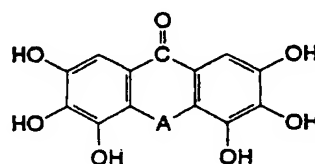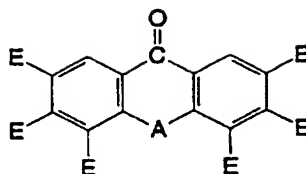Formula X6:

35

Formula X6E:

wherein A is oxygen, sulfur or NH and E is an ester.
Specific examples of such compounds include 2,3,4,5,6-
pentahydroxyxanthone; 2,3,4,5,6,-pentaacetoxyxanthone;
2,3,4,5,6,7,-hexahydroxyxanthone; 2,3,4,5,6,7,-
hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone;
2,3,4,5,6,-pentaacetoxyacridone; 2,3,4,5,6,7,-
hexahydroxyacridone; 2,3,4,5,6,7,-hexaacetoxyacridone;
2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6,-
pentaacetoxythioxanthone; 2,3,4,5,6,7,-
hexahydroxythioxanthone; and 2,3,4,5,6,7,-
hexaacetoxythioxanthone.

In the most preferred embodiments, the formula
X1 compounds are 2,3,4,5,6,-pentahydroxyxanthone and
esterified forms of this compound, including 2,3,4,5,6-
pentaacetoxyxanthone and 2,3,4,5,6,7-hexahydroxyxanthone
and esterified forms of this compound, including
2,3,4,5,6,7-hexaacetoxyxanthone.

The present invention thus includes compositions
for the treatment of microbial diseases such as malaria,
the compositions including a compound according to
formula X1. Also included in the present invention is a
method of inhibiting the growth of a microbial pathogen
comprising providing a sufficient amount of a formula X1
compound and contacting the microbial pathogen with this
compound. Such a method is applicable to inhibit
microbial growth *in vivo* and *in vitro*. In one aspect,
the present invention provides a method of treating a
patient having a microbial infection, the method
comprising administering to the patient a

therapeutically effective amount of a compound according
to formula X1.

Another aspect of the present invention is the
discovery that certain compounds having the underlying

5   xanthone ring structure depicted in formula X1 bind to,
and inhibit the polymerization of, heme. A number of
pathogens, including *Plasmodium*, a causative agent of
malaria, degrade hemoglobin to obtain amino acids, and
in so doing liberate toxic heme (Olliaro and Goldberg,

10   1995). To avoid the toxic effects of the liberated
heme, these pathogens polymerize the heme to form
hemozoin. The compounds disclosed herein which are
shown to inhibit heme polymerization may thus be used to
block heme polymerization and therefore to treat

15   infections caused by these pathogens. It is self-
evident that these heme complexing compounds may kill
pathogens by preventing these organisms from gaining
access to the host's supply of heme iron, or by causing
build-up of toxic levels of heme in the organism's

20   vacuole.

Compounds which are herein disclosed to inhibit
heme polymerization may be represented by the structure
     X - Y - Z
wherein X is a group capable of interacting with

25   the iron atom in heme;

Y is a planar aromatic system capable of
interacting with the porphyrin ring of heme through
overlapping pi-pi orbitals; and

Z represents one or more groups capable of

30   interacting with at least one carboxylate side group of
heme. In preferred embodiments, these compounds are
formula XH compounds, having a structure:
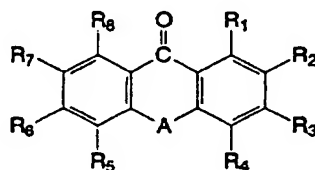
35

wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or haloaryl;

R_1-R_3 and R_6-R_8 are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloakyl), amino, ester and nitro groups and O-linked and C-linked carbohydrates;

at least one (and preferably both) of the R_4 and R_5 substituent pair is selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, gaunidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$; and

the other member of the R_4 and R_5 substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloakyl), amino, substituted amino, ester and nitro groups, O-linked and C-linked carbohydrates, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$. In preferred embodiments, both R_4 and R_5 are selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy

groups, and carboxamide derivatives of hydroxy, alkylhydroxy and alkoxyhydroxy groups.

Examples of formula XH compounds include 4,5-dihydroxyxanthone, 2,3,4-trihydroxyxanthone, 3,4,5,6-tetrahydroxyxanthone, 2,3,4,5,6-pentahydroxyxanthone, 1,3,5,6,7-pentahydroxyxanthone, 2,3,4,5,6,7-hexahydroxyxanthone; 4,5-bis-((β-diethylamino)ethoxy)xanthone; and 3,6-dihydroxy-4,5-bis-(piperidinomethyl)xanthone.

The present invention thus also includes compositions for the treatment of diseases such as malaria, which are caused by pathogens that polymerize heme, the compositions including a compound according to formula XH. Also included in the present invention is a method of inhibiting the growth of a such a pathogen comprising providing a sufficient amount of a formula XH compound and contacting the pathogen with this compound. Such a method is applicable to inhibit pathogen growth *in vivo* and *in vitro*. In one aspect, the present invention provides a method of treating a patient having malaria, the method comprising administering to the patient a therapeutically effective amount of a compound according to formula XH.

The invention also contemplates that formula X1 and XH compounds can be administered to patients in a pro-drug form, typically a corresponding substituted benzophenone which will react with oxidant radicals under physiological conditions to produce the active formula X1 or XH compound (Winter et al., 1996).

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 illustrates a proposed mechanism for the formation of 2,3,4,5,6-pentahydroxyxanthone from the metabolic activation of exifone by rufigallol within a red blood cell infected with the *Plasmodium* parasite.

Fig. 2 is a schematic depiction of hemoglobin
digestion (with the concomitant release of heme) by the
intracellular parasite *Plasmodium falciparum*.

5   Fig. 3 is a graph showing the inhibition of in
vitro heme polymerization by compound X5 (A=O)
(2,3,4,5,6-pentahydroxyxanthone). Heme and X5 (A=O)
concentrations were 25 μM. Open diamonds indicate heme
alone (control), filled diamonds represent heme and X5
10   (A=O) together.

Fig. 4 is a computer simulation of compound X6
(A=O) (2,3,4,5,6,7-hexahydroxyxanthone) complexing with
free heme.

15

Fig 5. Shows the structure of 45-DEAE-X (4,5-
bis-((β-diethylamino)ethoxy)xanthone) and formation of
the diprotonated form upon entry of this compound into
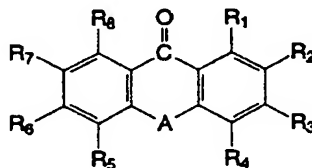the parasite digestive vacuole.

20

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

The phrase "a compound according to formula X1"
and "a xanthone derivative according to formula X1"
25   refer to a compound having the following structure:

30

wherein:

A is oxygen, substituted antimony (stibium),
sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl,
35   preferably lower alkyl or lower haloalkyl wherein
"lower" means 10 or fewer carbon atoms, aryl or
haloaryl; and

-11-

R_1-R_8 are independently selected from the group
consisting of H, OH, halogen, aryl, arylamine, alkyl,
substituted alkyl (such as alkoxy, alkylamine, alkylthio
and haloakyl), amino, ester and nitro groups and

5   O-linked and C-linked carbohydrates. In alternative
embodiments, an alkyl substituent is a lower alkyl.

References to compounds such as "an X5 compound"
refer to the compounds shown in the Summary of the
Invention section above. Where a particular substituent

10   in the formula is intended, it is given parenthetically.
For example X5 (A=O) refers to a compound according to
formula X5 wherein the A substituent is oxygen, i.e.,
2,3,4,5,6-pentahydroxyxanthone.

Compounds of the present invention which may be

15   used to inhibit heme polymerization (and therefore to
treat certain parasitic diseases such as malaria) are
referred to as "formula XH compounds". A formula XH
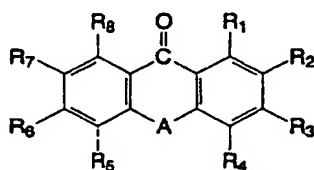compound is a compound broadly defined as:

X - Y - Z

20   wherein X is a group capable of interacting with
the iron atom in heme;

Y is a planar aromatic system capable of
interacting with the porphyrin ring of heme through
overlapping pi-pi orbitals; and

25   Z represents one or more groups capable of
interacting with at least one carboxylate side group of
heme.

In preferred embodiments, a formula XH compound
has the following structure:

30

35

wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or
5   haloaryl;

R_1-R_3 and R_6-R_8 are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloakyl), amino, ester and nitro groups
10   and O-linked and C-linked carbohydrates;

at least one member of the R_4 and R_5 substituent pair (and preferably both) is selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium,
15   alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$; and

20   the other member of the R_4 and R_5 substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloakyl), amino, substituted amino, ester and nitro groups, O-linked and
25   C-linked carbohydrates, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and
30   alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$.

As used herein, the term "alkyl" encompasses alkanes, alkenes and alkynes, including branched forms, isomers and stereoisomers. In certain embodiments, an
35   alkyl is a lower alkyl, meaning an alkyl having 10 or fewer carbon atoms.

The terms "ester" and "esterification" are used herein as ordinarily understood in the chemical arts,

see, for example, Morrison and Boyd, <u>Organic Chemistry</u>,
Allyn & Bacon, Inc., Boston, 1983, herein incorporated
by reference. Thus, an ester may be formed by, for
example, the combination of an alcohol and an organic
5   acid, with the concurrent elimination of water. The
process of forming an ester is termed "esterification."
For example, the formula X1 compound 2,3,4,5,6-
pentahydroxyxanthone may be esterified by reaction with
appropriate acid anhydrides resulting in the net
10   replacement of one or more hydroxyl substituents with
ester substituents including, but not limited to:
acetoxy ($OCOCH_3$); propionyloxy ($OCOCH_2CH_3$); and
butyryloxy $OCO(CH_2)_2CH_3$) substituents. Esters produced
in this manner may be generally represented by the
15   formula $OCO(CH_2)_nCH_3$ wherein n is zero or a positive
integer. In particular embodiments, the term "ester" as
used herein refers to an ester wherein n is 1-10.

     A "microbial pathogen" is a microorganism
capable of causing disease in an animal. The term
20   "microbial pathogen" includes bacterial, mycoplasmal,
fungal, helminth and protozoan organisms. "Protozoan
parasites" are a subclass of microbial pathogens, being
protozoan organisms that are capable of invading,
colonizing and, under appropriate conditions, causing
25   disease in an animal. Examples of protozoan parasites
include <i>Leishmania donovani</i>, <i>Plasmodium falciparum</i>,
<i>Giardia lamblia</i>, <i>Trypanosoma gambiense</i> and <i>Trypanasoma
cruzi</i>. <u>See generally</u>, Robbins <u>et al.</u>, Pathologic Basis
of Disease (Saunders, 1984) 273-75, 360-83.
30   A "microbial infection" is a disease caused by a
microbial pathogen.

     A compound having "anti-microbial activity" is a
compound that is capable of inhibiting the growth of a
microbial pathogen as determined in <i>in vivo</i> or <i>in vitro</i>
35   assays of the kind normally employed to determine
minimum inhibitory concentrations (MICs) or 50%
inhibitory concentrations (IC_{50}) of an antimicrobial
agent.

An "oxidant agent" is an agent having the
ability to produce or liberate free radical oxygen
species or to render parasites or their host cells more
susceptible to oxygen radical attack, or having the
capacity of oxidizing another compound. Examples of
oxidant agents in this general sense include ascorbic
acid, hydrogen peroxide, primaquine (or its metabolites)
and gamma radiation.

2. Methods

    A.   Methods for determining biological activity
The anti-parasitic activity of the compounds of
the present invention was determined using two different
parasites: *Plasmodium falciparum*, a causative agent of
malaria; and *Leishmania donovani*, a causative agent of
leishmaniasis. The activity of the compounds against
yeast was determined using *Candida albicans*.

    i.   Assay for anti-malarial activity
The D6 strain of *P. falciparum* was cultured
in Group A$^+$ human erythrocytes and suspended at a 3.3%
hematocrit in RPMI-1640 (Gibco, Grand Island, NY)
(containing 4g/L glucose, 50mg/L gentamicin and 10%
group A$^+$ human serum), buffered with 25mM HEPES and 25 mM
NaHCO$_3$ (Trager and Jensen, 1976). Cultures were
maintained at 37°C in a gas mixture of 5% oxygen, 5% CO$_2$,
and 90% nitrogen.

    The *in vitro* anti-malarial activities of
2,3,4,5,6-pentahydroxyxanthone and other formula X1
compounds were measured by the [^{3}H]-ethanolamine
incorporation method as described in Elabbadi et al.,
1992, with minor modifications. [^{3}H]-ethanolamine was
obtained from American Radiolabeled Chemicals, Inc., St.
Louis, MO. Experiments were conducted in 96 well plates
in a total volume of 200µl at a final red blood cell
concentration of 3.3% (v/v). An initial parasitemia of
0.2 to 0.5% was attained by addition of normal
uninfected red cells. Radiolabeled ethanolamine was
added after 48 hours of incubation and the experiment

was terminated after 72 hours by collecting the cells onto glass fiber filters with an automated multiwell harvester.

Stock solutions of the various formula X1
5   compounds were dissolved in DMSO at a concentration of 1 mM and diluted in complete medium (including 10% human serum) to provide 10X stock concentrations in the range of 1 to 10,000 nM. The concentration of the formula X1 compound giving 50% inhibition of label incorporation
10   (IC_{50}) relative to control (i.e., drug-free) conditions was calculated from the dose-response curve.

ii.   Assay for anti-leishmania activity
Leishmania donovani was cultivated in Schneider's medium (Gibco, Grand Island, NY) according
15   to the methods described by Grogl et al. (1992). The in vitro susceptibility of *L. donovani* to formula X1 compounds was determined using the radiolabeled thymidine uptake assay essentially as described by Grogl et al. (1992). Briefly, promastigotes were cultivated
20   at 25°C in Schneiders medium supplemented with 20% inactivated fetal calf serum and 100μg/mL of gentamicin. Cells were maintained in log phase by seeding at 1 x 10^6/mL with subculturing when cultured densities approached 4 x 10^7/mL before reaching their maximal
25   density. For the assay, early log phase promastigotes were counted on a hemacytometer and resuspended at a concentration of 1-2 x 10^6 cell/mL in assay media (Schneiders medium plus 10% fetal bovine serum). Ten-fold serial dilutions of each test compound were
30   prepared as described above and added to 180 μL of the parasite suspension. After incubation for 24 hours at 25°C, methyl-^{3}H-thymidine was added to each sample for a final concentration of 1-2 μCi per well. Each sample was then incubated for an additional 18 hours prior to
35   harvesting. After this incubation time, each sample was aspirated onto a filter mat, washed thoroughly with deionized water, dried and then counted in a scintillation counter with scintillation cocktail.

iii. Assay for anti-Candida activity

The minimum inhibitory concentration (MIC) of formula X1 compounds against a clinical isolate of *Candida albicans* was determined using the following method. As used herein MIC represents the concentration of formula X1 compound that completely inhibits growth of *Candida albicans* over the course of a 15-18 hour incubation period. The determination of this concentration is made by visual inspection; there is no visible growth in a tube containing the MIC of the formula X1 compound whereas visible growth is present in tubes containing sub-MIC concentrations of the compound.

Candida albicans was grown to midlog-phase in Luria-Bertani broth (10 grams Bacto-tryptone, 5 grams Bacto-yeast extract and 10 grams NaCl per liter) and then inoculated into sterile test tubes containing LB broth to an initial density of 10^3/ml. The formula X1 compound to be tested is dissolved in dimethylsulfoxide (DMSO) at a concentration of 10mM and added to each tube at serial dilutions (1μM, 10μM, 25μM, 50μM, 100μM and 0μM). The tubes are incubated at 35°C for 15-18 hours and then visually inspected.

B.   Heme Assays
i.   In Vitro Heme Polymerization Assay

Heme polymerization was carried out in 0.02 M phosphate buffer, pH 5.2 at 37°C in the absence of proteins. A 10 mM stock solution of hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 1 hour to effect complete dissolution. Xanthones were dissolved in dimethylformamide at 10 mM and diluted into 10 ml of pre-warmed phosphate solution to a final concentration of 25 μM. Polymerization was initiated by addition of 25 μl of the hemin stock solution to the test sample to yield a final concentration of 25 μM heme. 25 μl of dimethylformamide was added to the control sample. After 7, 30, 60, 120 and 210 minutes of incubation at 37°C, a 1 ml aliquot was withdrawn, transferred into an Eppendorf tube, and

centrifuged at 14000g for 2 minutes at room temperature to pellet the precipitate. The soluble fraction was then transferred to a semi-microcuvette (polymethylacrylate, VWR), and its absorption was

5 measured at 360 nm against a blank of the test compound in buffer. Control experiments indicated that (I) the pH of the phosphate solution did not change upon addition of the reagents or during the polymerization process, and (ii) the amount of dimethylformamide used

10 in this assay did not significantly affect the rate of polymerization. To estimate the effect of test compounds on heme polymerization at a given time of incubation, the percentage of soluble hemin remaining in the sample was calculated using the following formula:

15

$$\% \text{ sol. hemin} = [A_{(drug+hemin)t} - A_{(drug)t}] / [A_{(hemin)t=0}] \times 100\%$$

where $A_{(drug+hemin)t}$ is the absorption (360 nm) of the soluble fraction in the drug-hemin sample after various

20 times of incubation; $A_{(drug)t}$ is the absorption of the drug alone; and $A_{(hemin)t=0}$ is the absorption of the hemin control sample (25 μM) measured immediately upon addition of the hemin stock solution.

The dose-dependent inhibition of heme

25 polymerization was evaluated as described above except the concentration of each drug was varied in the range of 0 to 1 mM. The reactions were allowed to proceed for 2 hours in a 37°C waterbath. After incubation, the polymer was pelleted as described above and the

30 absorption (360 nm) of each soluble fraction was measured against a blank containing the drug alone in buffer. The IC_{50} values were determined by nonlinear regression analysis of the dose-response curves of percent inhibition of heme polymerization vs. drug

35 concentration.

-18-

3.   <u>Production of 2,3,4,5,6-pentahydroxyxanthone
      inparasitized erythrocytes treated with
      rufigallol and exifone</u>

              As disclosed in Winter et al. (1996), rufigallol
5   (1,2,3,5,6,7,-hexahydroxy-9,10-anthraquinone) is a
     potent anti-parasitic agent and, when rufigallol is
     combined with exifone (2,3,3',4,4',5'-
     hexahydroxybenzophenone), a synergistic effect is
     observed. The synergy between rufigallol and exifone is
10   noted to produce about a 350-fold increase in potency
     against malaria *Plasmodium* parasites.

              One aspect of the present invention is the
     discovery that rufigallol and exifone interact in the
     parasitized erythrocyte to yield 2,3,4,5,6-
15   pentahydroxyxanthone, and that this compound is a potent
     anti-malarial agent. Fig. 1 shows a possible mechanism
     by which 2,3,4,5,6-pentahydroxyxanthone could be
     produced when rufigallol and exifone are present in a
     parasitized erythrocyte. Basically, rufigallol is
20   proposed to enter the parasitized erythrocyte, leading
     to the formation of hydrogen peroxide in a manner
     similar to the well-documented redox cycling behavior of
     hydroxynaphthoquinones. In the presence of large
     quantities of adventitious iron or iron chelates, such
25   as heme, (liberated as a result of the *Plasmodium*
     parasite digesting hemoglobin, Atamna and Ginsburg,
     1993), hydrogen peroxide is readily decomposed to
     hydroxyl radicals (Goldstein et al., 1993; Aust et al.,
     1985). These highly reactive radicals are proposed to
30   attack exifone and transform the diphenyl compound into
     2,3,4,5,6-pentahydroxyxanthone.

              As reported in U.S. application serial No.
     08/520,694, the anti-malarial activity of exifone can be
     potentiated by a very wide range of oxidant agents,
35   including ascorbic acid, artemisinin and doxorubicin.
     This observation is consistent with the mechanism
     proposed above. The production of 2,3,4,5,6-
     pentahydroxyxanthone in the proposed reaction scheme was
   confirmed by incubating exifone with ascorbic acid in

the presence of iron salt and oxygen in a buffered
solution at 37-40°C (the "Udenfriend system," Brodie
et al., 1954; Maisant et al., 1983; Udenfriend et al.,
1954). Samples were removed from the reaction at
5   various time points, lyophilized and extracted with
acetone. The solubilized products were then methylated
by addition of excess potassium carbonate and
dimethylsulfate in acetone and analyzed by gas
chromatography-mass spectrometry. A peak corresponding
10   to the methoxy derivative of 2,3,4,5,6-
pentahydroxyxanthone was detected.

4.   Synthesis and anti-microbial activity of
15   2,3,4,5,6-pentahydroxyxanthone
         The formula X1 compound 2,3,4,5,6-
pentahydroxyxanthone is produced using the following
method.
         A mixture of 1,2,3-Trimethoxybenzene (1.48g) and
20   2-hydroxy-3,4,5-trimethoxybenzoic acid (2.00g) is
stirred in 40ml of ~9% solution of P_2O_5 in
methanesulfonic acid at room temperature in a stoppered
flask for 4 hours. The 2-hydroxy-3,4,5-
trimethoxybenzoic acid was obtained by the method of
25   Mayer and Fikentscher (Mayer and Fikentscher (1956)
Chem. Ber. 89:511) from 3,4,5-trimethoxybenzoic acid by
bromination and then copper-catalyzed replacement of
bromine (by OH) of 2-bromo-3,4,5-trimethoxybenzoic acid.
The resultant orange mixture is poured onto crushed ice
30   (500ml) producing an unfilterable gummy precipitate.
This crude product is then subjected to base-catalyzed
ring closure by heating in a beaker in 100ml of 40%
ethanol and 10ml of 10N NaOH just below boiling point.
As the mixture reaches 80°C, a white flocculent product
35   appears. The temperature is maintained just below the
boiling point and the volume is kept constant by
addition of water. After 5 hours, the supernatant is
bright yellow and a mass of the precipitate has formed.
Heating is continued for 4 more hours. Cooling,

-20-

filtering (by suction) and washing with water afforded
1.37g of analytically pure 2,3,4,5,6-
pentamethoxyxanthone (yield approximately 45% relative
to benzoic acid). This base-catalyzed ring closure is
5   illustrated below:
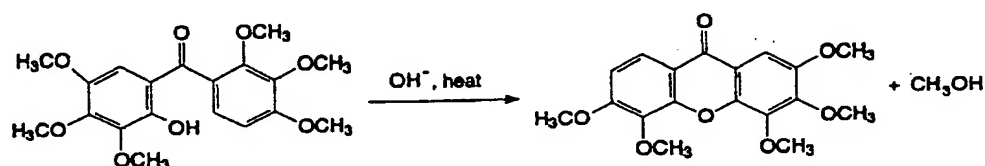
2,3,4,5,6-pentahydroxyxanthone is then obtained by boron
tribromide treatment (200 ml of a 0.8 M solution in
CH_2Cl_2) as the pentamethyl ether (0.45 g) is stirred at
room temperature for 24 hours. After this period, the
10   solution is poured into 100 ml of water and stirred for
approximately 45 minutes before the precipitate is
collected by centrifugation. The supernatant is then
decanted, the precipitate is shaken with water and
centrifuged again. The final product is obtained by
15   freeze-drying of the wet precipitate to produce an
orange powder (0.290g, 81%).
        The anti-malarial activity of 2,3,4,5,6-
pentahydroxyxanthone was determined by the method
described above. The IC_{50} was determined to be 0.4-0.5
20   μM. Chloroquine, a standard anti-malarial agent has an
IC_{50} in this assay system of approximately 0.02 μM.
        The anti-leishmanial activity of 2,3,4,5,6-
pentahydroxyxanthone was determined by the method
described above. The IC_{50} was determined to be
25   approximately 5 μM or 0.001 mg/ml. Mangostin, a
naturally occurring xanthone, exhibited an IC_{50} of 1 μM
(or 0.00041 mg/ml) in this same system. Grögl et al.
(1992) report that two commonly used anti-leishmanial
drugs, Pentostam and Glucantine, have IC_{50} values in the
30   range of approximately 0.1 - 2 mg/ml.
        The MIC of 2,3,4,5,6-pentahydroxyxanthone
against Candida albicans, determined using the method

described above was found to be approximately 37.5 μM.
This corresponds to an IC_{50} of approximately 10 μg/ml.

5.   Synthesis and anti-microbial activity of
        2,3,4,5,6-pentaacetoxyxanthone
        Although 2,3,4,5,6-pentahydroxyxanthone was
found to have potent anti-malarial activity, the
inventors postulated that the highly acidic nature of
the 3 and 6 hydroxy groups of this compound (i.e. the R_3
and R_6 positions as shown in formula X1) could lead these
groups to be highly ionized at physiological pH values.
Such ionization would likely reduce the rate at which
the compound could cross biological membranes, thereby
lowering the uptake of the compound into parasitized
erythrocytes. Accordingly, two derivatives of
2,3,4,5,6-pentahydroxyxanthone were produced which were
expected to be more stable and uncharged above neutral
pH: a pentacetoxy (i.e. esterified) derivative,
2,3,4,5,6-pentaacetoxyxanthone, as well as a methoxy
(i.e. methyl ether) derivative, 2,3,4,5,6-
pentamethoxyxanthone. The activity of these two
derivatives against *P. falciparum* was measured.
        As shown in Table 1, the addition of the ether
(methoxy) groups essentially eliminated the anti-
malarial activity of the compound, resulting in an IC_{50}
of >100 μM. This reduction in activity is believed to
be attributable to the extreme stability of the methoxy
groups; the methoxy group is less amenable to enzymatic
cleavage under physiological conditions.
        The pentaacetoxy derivative was produced by
heating 2,3,4,5,6,-pentahydroxyxanthone in acetic
anhydride in the presence of a catalytic amount of
sulfuric acid, followed by recrystallization. In
contrast to the methoxy derivative, the esterified
2,3,4,5,6,-pentaacetoxyxanthone was several times more
potent than 2,3,4,5,6-pentahydroxyxanthone (exhibiting
an IC_{50} of approximately 0.075 μM). The enhanced
activity of the esterified compound is postulated to be
due to the ability of the compound to cross membranes

(due to its neutral charge at physiological pH). Esters
are also known to be amenable to enzymatic cleavage
under physiological conditions. Accordingly, it is
expected that the pentaacetoxyxanthone enters the cell

5   where it is enzymatically cleaved to produce
pentahydroxyxanthone.

6.   Synthesis and anti-microbial activity of
        2,3,4,5,6,7,-hexahydroxyxanthone

10   The newly discovered anti-malarial activity of
2,3,4,5,6-pentahydroxyxanthone prompted the
investigation of other xanthones and related compounds.
One such related compound was 2,3,4,5,6,7,-
hexahydroxyxanthone which was prepared using the

15 following method:

        2-hydroxy-3,4,5-trimethoxybenzoic acid (1.14g,
0.005 mol) and 1,2,3,4-tetramethoxybenzene (0.99g, 0.005
mol) and 25 ml of a 9% solution of P_2O_5 in
methanesulfonic acid were shaken in a 50ml cylindrical

20 glass tube with a Teflon-lined screw-cap at room
temperature for 54 hours. The dark orange mixture was
then poured onto crushed ice (150 ml). After melting,
the product was extracted with methylene chloride
(3 x 40 ml). After removal of the solvent, the residue

25 was chromatographed on silica gel (30 g) with CH_2Cl_2. Of
the three fractions obtained (the eluent was monitored
by thin-layer chromatography), the middle one was
uniform and left pure 2-hydroxy-,3,4,5,2',3',4',5'-
heptamethoxybenzophenone (0.51 g, 25%) as a yellow oil

30 upon evaporation of the solvent. This was dissolved in
100 ml 75% alcohol whereafter 5 ml of 10N NaOH were
added and the mixture was heated to boiling in a beaker
for three hours; the volume was kept at 100 ml by
occasional addition of water. The mixture was then

35 transferred to a 250 ml round bottom flask and refluxed
for another 17 hours. After cooling, suction filtration
yielded 0.36 g of 2,3,4,5,6,7-hexamethoxyxanthone as a
white product (small needles, matted, 77%). In a

deprotection procedure, similar to the one described
above for pentamethoxyxanthone, 0.42 g of the
hexamethoxyxanthone produced 0.296 g of
hexahydroxyxanthone (91%) as a pale yellow powder. It
5   was found advantageous to circumvent the need for
centrifugation by stirring the methylene chloride-water
mixture (from the quenching of the BBr_3-solution) in a
wide-mouthed container for several hours, leading to the
evaporation of the methylene chloride; the mixture is
10   then easily filterable.

The antimalarial activity of 2,3,4,5,6,7-
hexahydroxyxanthone was determined by the method
described above. The IC_{50} was determined to be 0.075 μM.
The IC_{50} of this compound against *Leishmania* was
15   determined to be approximately 5 μM. The MIC of the
compound against *Candida albicans* was determined to be
approximately 37.5 μM, corresponding to an IC_{50} of
approximately 10 μg/ml.

20   7.   Scope of formula X1 compounds

The inventors have discovered that a wide range
of compounds related to 2,3,4,5,6-pentahydroxyxanthone
have anti-microbial activity. These compounds can be
represented by the formula X1 shown below.
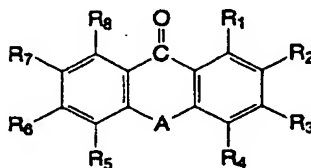
25

30

wherein:

A is oxygen, substituted antimony (stibium),
sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl,
aryl or haloaryl; and
35   R_1-R_8 are independently selected from the group
consisting of H, OH, halogen, aryl, arylamine, alkyl,
substituted alkyl (such as alkylamine, alkylthio and

haloakyl), amino, ester and nitro groups and O-linked
and C-linked carbohydrates.

The activities of various formula X1 compounds
against the *Plasmodium falciparum* parasite are shown in
5   Table 1. The activities of various formula X1 compounds
against *Leishmania donovani* are shown in Table 2. For
comparison, Table 2 also shows the activity of
stibogluconate, a standard anti-leishmanial. Other
examples of specific formula X1 compounds are
10   illustrated below:
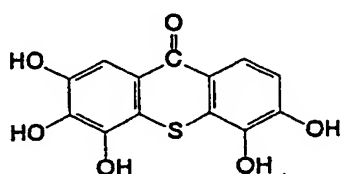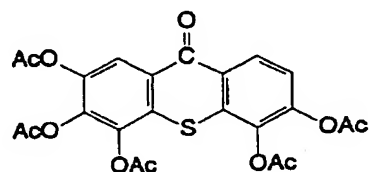
2,3,4,5,6-pentahydroxythioxanthone   2,3,4,5,6-pentaacetoxythioxanthone
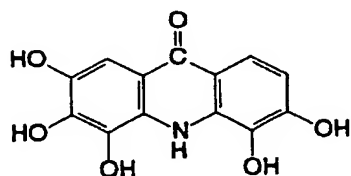
2,3,4,5,6-pentahydroxyacridone   2,3,4,5,6-pentaacetoxyacridone
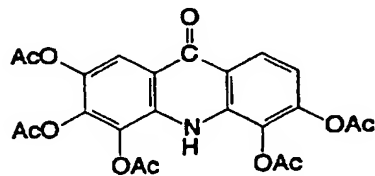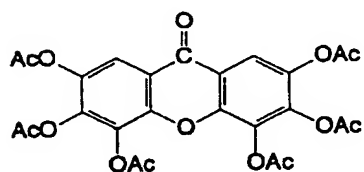
2,3,4,5,6,7-hexaacetoxyxanthone   2,3,4,5,6,7-hexahydroxythioxanthone
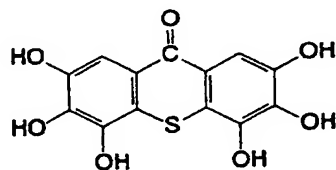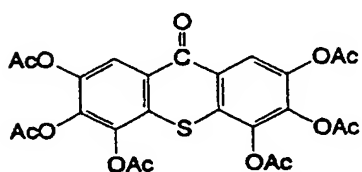
2,3,4,5,6,7-hexaacetoxythioxanthone   2,3,4,5,6,7-hexaacetoxyacridone
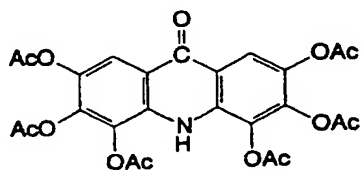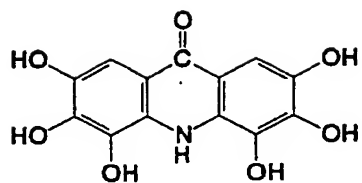
2,3,4,5,6,7-hexahydroxyacridone
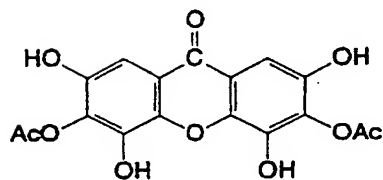
2,4,6,7-tetrahydroxy-3,6-acetoxyxanthone
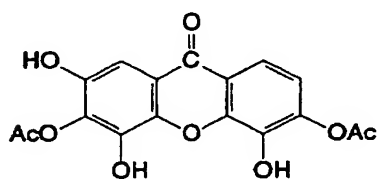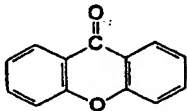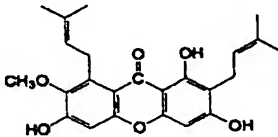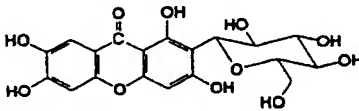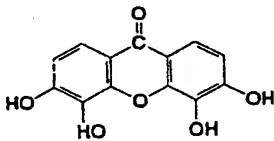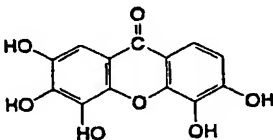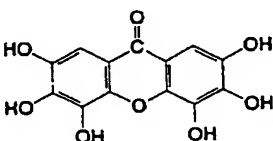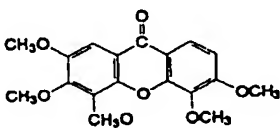
2,4,5-trihydroxy-3,6-acetoxyxanthone

5

10

15

20

25

Table 1

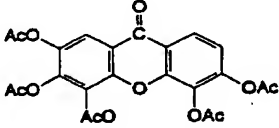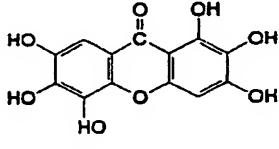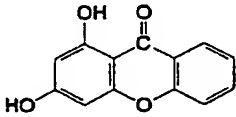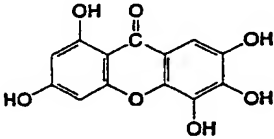| Compound Name	Xanthone Structure	IC$_{50}$, μM vs. Plasmodium
Xanthone		>10
Mangostin		5
Mangiferin		50
3,4,5,6,-Tetrahdroxy-xanthone		10
2,3,4,5,6-Pentahydroxy-xanthone		0.4 to 0.5
2,3,4,5,6,7-hexahydroxy-xanthone		0.075
2,3,4,5,6-Pentamethoxy-xanthone		>100
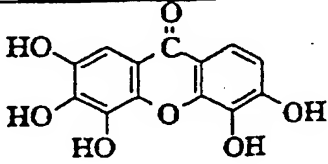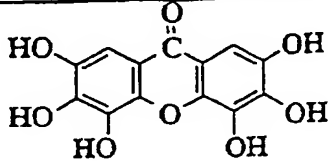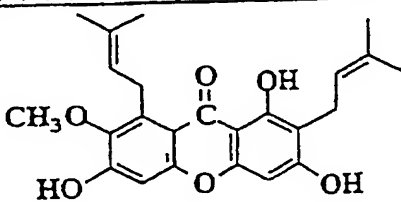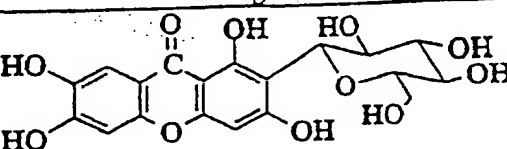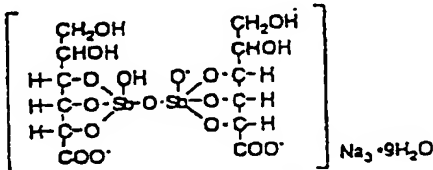
Compound Name	Xanthone Structure	IC$_{50}$, μM vs. *Plasmodium*
2,3,4,5,6-Penta-acetoxyxanthone		0.075
1,2,3,5,6,7-Hexahydroxy-xanthone		25-50
1,3-dihydroxyxanthone		> 100μM
1,3,5,6,7-pentahydroxy-xanthone		1μM

5

10

Table 2

Compound	IC_{50}, mg/ml vs. Leishmania
2,3,4,5,6-Pentahydroxyxanthone "X5"	0.0015
2,3,4,5,6,7-hexahydroxyxanthone "X6"	0.0015
Mangostin	0.00041
Mangiferin	>0.05
Sodium Antimonyl (V) Gluconate "Stibogluconate" Stibogluconate	0.1 to 1.0*

*taken from literature values

8.   Sources of formula X1 compounds and preferred
        method of synthesis

        Many xanthones and xanthone derivatives can be
5   purchased commercially from sources including: ICN
    Biomedicals, Irvine, California, U.S.A.; Sigma Chemical
    Company, St. Louis, Missouri, U.S.A.; Aldrich Chemical
    Company, Milwaukee, Wisconisin, U.S.A.; and Janssen
    Chimica (Belgium). In addition, many xanthones are
10 naturally occurring compounds which can be purified by
    methods such as those described in Hostettmann et al.
    (1995).

        A.   General method of xanthone synthesis

        Xanthones according to the present invention may
15 be synthesized by the general method described above,
    for the synthesis of 2,3,4,5,6,7-hexahydroxyxanthone and
    2,3,4,5,6-pentahydroxyxanthone. Essentially, this
    method comprises subjecting an o-hydroxy-o'-methoxy-
    benzophenone to base treatment (e.g., aqueous sodium
20 hydroxide), which leads to the formation of the central
    oxygen-bridged ring; the o-phenoxide (from the o-
    hydroxyl in basic medium) then replaces the methoxide on
    the other ring by nucleophilic substitution. The net
    effect is expulsion of CH_3O^-, and the formation of a
25 diphenyl ether. Since the two phenyl rings are already
    linked by a carbonyl group, a xanthone is obtained. The
    o-OH,o'-OCH$_3$ groupings are required for this reaction;
    although the methyl could be replaced with other groups,
    this is not likely to be of any advantage since methyl
30 ethers are readily available. However, other
    substituents can be present in the two aromatic rings of
    the benzophenones. For example, for the synthesis of
    the penta- and hexa-hydroxyxanthones described above,
    these other substituents were methoxy groups.

35   The benzophenones used in synthesizing the
    xanthones as described above may be obtained by
    combining substituted benzoic acids and methoxybenzenes
   by a condensation or other coupling procedure. In an

exemplary condensation procedure, the benzoic acid
carries an o-hydroxy group:
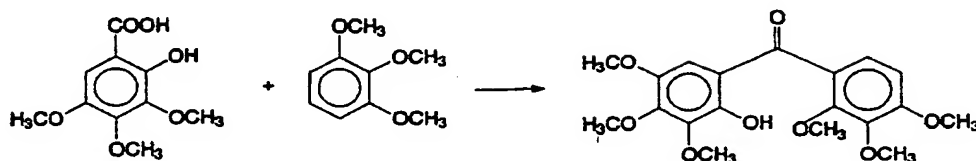
This coupling can be achieved by condensation in
polyphosphoric acid or a mixture of phosphorus pentoxide
5   and methanesulfonic acid. Alternatively, benzophenones
may be synthesized by Friedel-Crafts acylation (of a
benzoyl chloride and a polymethoxybenzene), or by the
Hoesch synthesis, or by reaction of a benzoyl chloride
with an appropriately metalated (e.g., lithiated)
10   aromatic.
         In particular cases, additional substituents may
be introduced into the benzophenone after the
benzophenone has been synthesized.
         Alternatively, xanthones may be derived from
15   benzophenones by oxidative cyclization. This method
essentially requires an o-hydroxy group on one ring and
a free position (occupied by H) on the other ring.
Oxidation (e.g., with $K_3[Fe(CN)_6]$, or $KMnO_4$) produces an
oxygen bridge with the elimination of 2H.

20

25
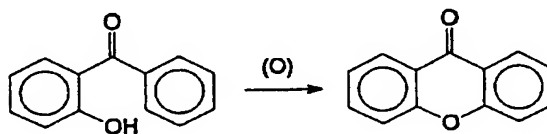
B.   Synthesis of Thioxanthones
         Thioxanthones may be obtained by a number of
methods. Exemplary syntheses include: (1) combining an
o-mercaptobenzoic acid with a halobenzene (preferably
30   iodo or bromo); and (2) combining an o-halobenzoic acid
(preferably either bromo or iodo) with a
mercaptobenzene. The intermediate diphenylsulfide
produced in each case is then condensed to yield the

-31-

required thioxanthone as illustrated in the following schematic:

5
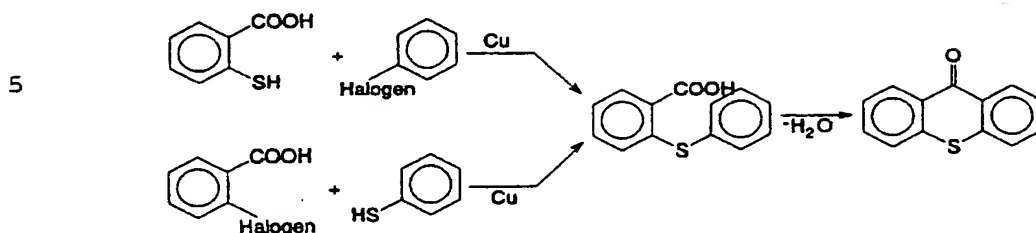
10   Methods of synthesizing thioxanthones using this general reaction scheme are described in Hollis-Showalter et al., _J. Med. Chem._ 31, 1527 (1988).

        C.   Synthesis of Acridones

        Acridones may be synthesized by a number of
15 different methods. The following methods are exemplary and are well known in the art.
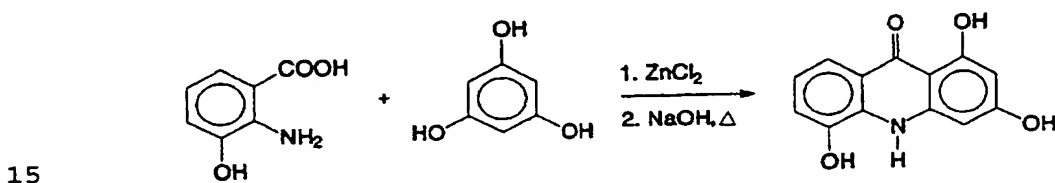
        Acridones may be formed from
o-nitrobenzophenones, which are reduced to obtain
o-aminobenzophenones which are in turn cyclized with
20 either o'-methoxy or o'-hydroxy groups to produce the acridones. The o-nitrobenzophenones which are used as starting materials may be obtained either by Friedel-Crafts acylation of phenols or methoxybenzenes using o-nitrobenzoylchlorides, or by direct nitration of
25 benzophenones, or by coupling of lithiated arenes with o-nitrobenzol chlorides (e.g., as described by Parkham et al., _Journal of Organic Chemistry_ 46, 1057 (1981).
An exemplary synthesis is illustrated below:

30

35
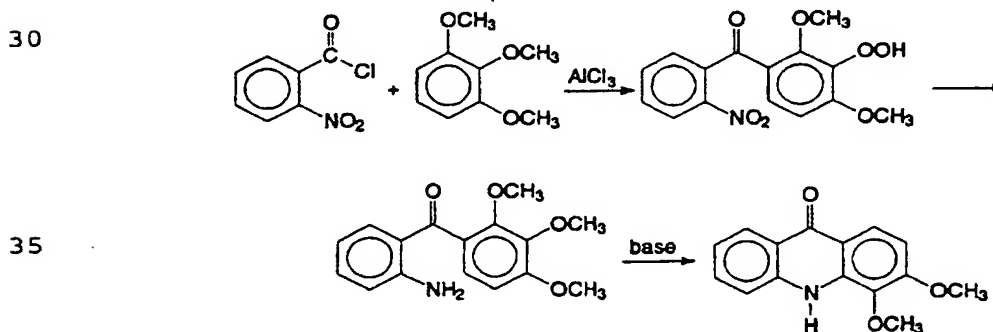
Alternatively, o-nitrobenzophenones may be formed by
coupling of 2-methyl-3,1-benzoxazin-4-ones (from
o-aminobenzoic acid by heating with acetic anhydride)
with aromatic Grignard reagents (e.g., Adams et al.

5   J.C.S. Perkin Trans I 2089 (1976)).

        Alternatively, acridones may be produced by zinc
chloride catalyzed condensation of hydroxyanthranilic
acids and polyhydroxybenzenes (such as described by
Bahar et al., Phytochemistry 21, 2729 (1982)) and

10   illustrated in the following scheme:

15

        Acridones may also be formed by cycloaddition of
derivatives of anthranilic acids with dehydrobenzenes
such as described by Khanapure et al., Tetrahedron

20   Letters 31:2869 (1990).

25
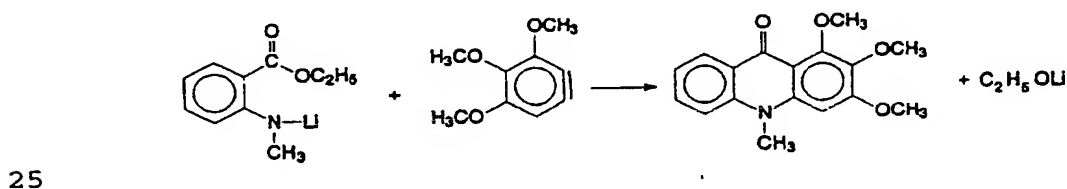
D.   Deprotection

        Deprotection of polymethoxyxanthones,

30   polymethyoxythioxanthones or polymethoxyacridones may be
achieved in a number of ways, including treating with
either hydriodic acid or with a methylene chloride
solution of boron tribromide, and hydrolysis of the
intermediate boron-phenoxy compound.

35

9.   Activity of formula X1 compounds

        The formula X1 compounds according to the
present invention are useful in inhibiting the growth of
microbial pathogens, including protozoan parasites (for
5   example, *Plasmodium* sp. and *Leishmania* sp.) and yeast
(for example, *Candida albicans*). Thus, one aspect of
the present invention is a method of inhibiting the
growth of a microbial pathogen by contacting the
microbial pathogen with a formula X1 compound. In this
10   context, it is, of course, necessary to contact the
microbial pathogen with a sufficient amount of the
formula X1 compound to inhibit growth of the pathogen.
One skilled in the art will readily appreciate that the
amount of compound sufficient to inhibit the growth of
15   the microbial pathogen will vary according to the
formula X1 compounds selected, the target microbial
pathogen and the environment in which the microbial
pathogen is growing. Standard methods are available for
determining the IC_{50} concentration of formula X1
20   compounds for microbial pathogens *in vitro*.
Alternatively, ED_{50} values may be determined in an
animal. See Munson, Principles of Pharmacology (Chapman
and Hall, 1995) Chapter 1. Exemplary IC_{50} values
(showing activities against *Plasmodium* and *Leishmania*,
25   respectively) are presented in Tables 1 and 2. These
values relate to the inhibition of a microbial pathogen
grown *in vitro*. Contacting the microbial pathogen with
a compound according to formula X1 may also be performed
in vivo where necessary to inhibit the growth of
30   microbial pathogens under physiological conditions.
Section 11 below ("Pharmaceutical Compositions")
addresses compositions and dosages appropriate for
inhibiting the growth of microbial pathogens in such
circumstances.

35   10.   Heme Polymerization and Formula XH Compounds

        Certain parasites, including *Plasmodium* spp. and
Schistosoma spp., obtain amino acids for growth by
degrading hemoglobin from the red blood cells of the

infected host. In the case of the malarial parasite, degradation of hemoglobin takes place in the parasite's digestive vacuole, which is an acidic proteolytic compartment essential to the metabolism of the parasite (see Fig. 2). As the hemoglobin is broken down, free toxic heme is released. To prevent the build-up of toxic heme, the parasites polymerize the heme for storage in a non-toxic form called hemozoin.

While the formula X1 compounds of the present invention exhibit anti-microbial activity against a range of pathogens, it has now been discovered that a certain sub-group of these compounds form complexes with free heme, which results in the inhibition of heme polymerization. In turn, this leads to the accumulation of toxic heme in the parasite's digestive vacuole and ultimately to the death of the parasite. These compounds, include X5 (A=O) and X6 (A=O) and belong to a group related to formula X1 compounds which are referred to herein as "formula XH compounds". Formula HX compounds may be particularly effective against those parasites, including *Plasmodium* and *Schistosoma*, which rely on hemoglobin catabolism to survive in the infected host or must rely on the host's heme iron reserves for synthesis of critical ferroproteins.

Table 3 below shows the ability of a range of formula X1 compounds to inhibit heme polymerization, as determined using the simple in vitro heme polymerization assay described above. Under the conditions of this assay, heme polymerization was found to be pH dependent (polymerization required a pH of between 4.5 and 5.5). Polymerization occurred spontaneously and was more than 95% complete within 2 hours of commencement of incubation with the compound X5 (A=O)(see Fig. 3).

The IC_{50} values shown in Table 3 are the average of at least two independent determinations of full dose-response curves. Xanthone and the tested monohydroxyxanthones did not exhibit any inhibitory activity in this assay. Moderate inhibitory activity
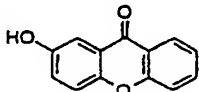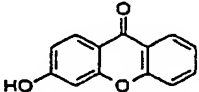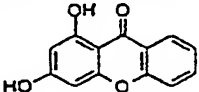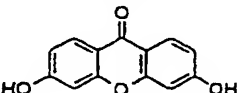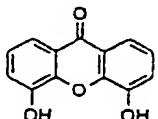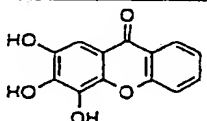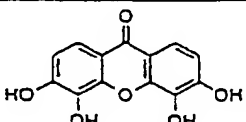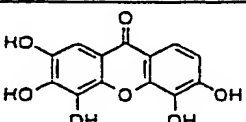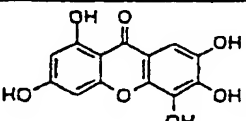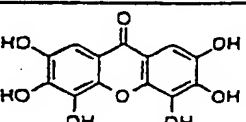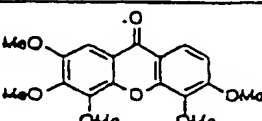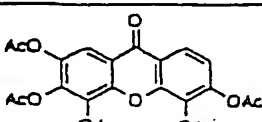
(i.e., IC_{50} 8-20 μM) was observed for the compounds
bearing a single hydroxy group at either 4- or 5-
position, whereas the greatest activity was observed for
xanthones containing hydroxy groups at both positions.

5   For example, 2,3,4-trihydroxyxanthone exhibited an IC_{50}
of 16.5 μM, while 2,3,4,5,6-pentahydroxyxanthone (X5,
A=O) yielded a value of 1.2 μM. Consistent with this
structure-activity profile, the 4,5-hydroxylated
xanthones also exhibited the most pronounced in vitro

10   antimalarial activity. Furthermore, pentamethoxy-X5 and
pentaacetyl-X5 were inactive in this assay, though the
latter was shown to be a potent antimalarial agent.
Presumably, pentaacetyl-X5 is hydrolysable in infected
red blood cells by a non-specific esterase, whereas

15   pentamethoxy-X5 is not.

Table 3   - 36 -

Compound name	Compound structure	IC_{50} µM, *P. falciparum* clone D6	IC_{50}, µM *in vitro* heme polymerization
2-hydroxyxanthone		50	>1000
3-hydroxyxanthone		>100	>1000
1,3-dihydroxyxanthone		75	>1000
3,6-dihydroxyxanthone		>100	>500
4,5-dihydroxyxanthone		16	14
2,3,4-trihydroxyxanthone		40	17
3,4,5,6-tetrahydroxyxanthone		5	2.5
2,3,4,5,6-pentahydroxyxanthone (X5)		0.4	1.2
1,3,5,6,7-pentahydroxyxanthone		1	9
2,3,4,5,6,7-hexahydroxyxanthone (X6)		0.1	1.4
2,3,4,5,6-pentamethoxyxanthone		>100	>1000
2,3,4,5,6-pentaacetylxanthone		0.075	>1000

These findings suggest that X5 (A=O) and other
compounds shown in Table 3 form soluble complexes with
heme monomers or oligomers and interfere with hemozoin
5   formation. Such action may result in the death of the
parasite by one of several mechanisms, including
preventing detoxification of free heme, starving the
parasite for iron, or increasing the osmotic pressure
within the parasite digestive vacuole. The relative
10   abilities of these compounds to inhibit in vitro heme
polymerization are in good correlation with their in
vitro antimalarial activities, and are indicative of the
following structure-activity relationships: (I) in
general, a higher degree of hydroxylation is favored for
15   the inhibitory activity; and (ii) hydroxylation at 4-
and 5-positions appears to be central to full activity.
Based on these observations, a model for the interaction
of these compounds is presented in Fig. 4. This model
shows the interaction of X6 (A=O) with heme and serves
20   to illustrate the following interactions: (1) between
the heme iron and the carbonyl oxygen; (2) between the
two planar aromatic systems; and (3) between the
carboxylate side groups of the heme and the 4- and 5-
position hydroxyls of the xanthone. These interactions
25   may take any form of known chemical interaction,
including covalent bonding, hydrogen bonding, ionic
bonding, and polar and nonpolar bonding. Moreover, this
model predicts that chemical modifications at the 4-
and/or 5-positions which improve association with the
30   heme carboxylate groups will result in even greater
antimalarial activity.

Accordingly, Formula XH compounds can be defined
as compounds which inhibit heme polymerization and which
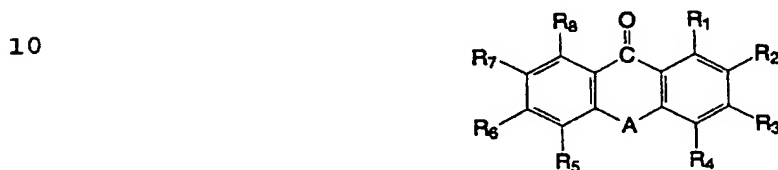have the following structure:
35   X - Y - Z

wherein X is a group capable of interacting with
the iron atom in heme;

Y is a planar aromatic system capable of interacting with the porphyrin ring of heme through overlapping pi-pi orbitals; and

Z represents one or more groups capable of interacting with at least one carboxylate side group of heme.

In preferred embodiments, a formula XH compound has the following structure:

wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or haloaryl;

R_1-R_3 and R_6-R_8 are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloakyl), amino, ester and nitro groups and O-linked and C-linked carbohydrates;

at least one of the R_4 and R_5 substituent pair is selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$; and

the other member of the R_4 and R_5 substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloakyl), amino, substituted amino, ester and nitro groups, O-linked and

C-linked carbohydrates, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine,

5   azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$.

Preferably, both R_4 and R_5 are selected from the group consisting of amino, substituted amino,

10   alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy

15   groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$.

Examples of formula XH compounds include: 4,5-dihydroxyxanthone, , 2,3,4-trihydroxyxanthone, 3,4,5,6-tetrahydroxyxanthone, 2,3,4,5,6-pentahydroxyxanthone, 1,3,5,6,7-pentahydroxyxanthone, 2,3,4,5,6,7-

20   hexahydroxyxanthone. Methods of synthesizing these compounds are known in the art and are described in a representative manner above in relation to formula X1 compounds (it will be appreciated that there is significant overlap between formula X1 and formula XH

25   compounds).

Based on the mechanism proposed for the interaction of formula XH compounds with heme, the formula XH compound 4,5-bis-((β-diethylamino)ethoxy)xanthone (45-DEA-X) is anticipated

30   to be particularly effective in complexing with heme. 4,5-DEAE-X, illustrated in Fig. 5, is a diprotic base which upon entry into the acidic vacuole becomes positively charged, effectively "trapping" the drug within this compartment where it will complex with heme.

35   The positively charged residues are arranged to be in opposition to the heme carboxylate side chains so as to facilitate formation of a soluble heme:xanthone complex (the ionic nature of the trapped xanthone will also

maintain the drug:heme complex in solution).   4,5-DEAE-X
may be readily prepared from 4,5-dihydroxyxanthone
(which as described above, is synthesized by base-
catalyzed cyclization of the appropriate ortho-hydroxy-
5   methoxylated-benzophenone).   To produce 4,5-DEAE-X,
4,5-Di-hydroxyxanthone is then reacted under basic
conditions with ethylene dibromide to yield 4,5-bis-(β-
bromoethoxy)-xanthone.   The latter is then reacted with
diethylamine to yield the desired product.   The shorter
10   dimethylaminomethoxy derivative is also easily available
from 4,5-dihydroxyxanthone through reaction with
Eschenmoser's salt.
        The present invention thus encompasses the use
of formula XH compounds to inhibit heme polymerization
15   and to inhibit the growth of those pathogens which
polymerize heme, such as *Plasmodium*.   It is also
apparent that the formula XH heme-complexing compounds
may be used to treat infections caused by pathogens
which require access to the host heme iron reserves for
20   survival.

11.   Pharmaceutical compositions
        Formula X1 and XH compounds having anti-
microbial activity are administered to patients in
25   conventional dosage forms prepared by combining an
appropriate dose of the compound with standard
pharmaceutical carriers.   Suitable pharmaceutical
carriers may be, for example, solids or liquids.
Suitable solid carriers include lactose, magnesium
30   stearate, terra alba, sucrose, talc, stearic acid,
gelatin, agar, pectin, acacia and cocoa butter.   The
amount of solid carrier will vary widely depending on
which carrier is selected, but preferably will be from
about 25mg to about 1g.   Suitable liquid carriers
35   include syrup, peanut oil, olive oil, sesame oil,
propylene glycol, polyethylene glycol and water.   The
carrier or diluent may also include time delay material
well known to the art such as, for example, glyceryl,

monostearate or glycerol distearate, either alone or
with a wax. The foregoing examples of suitable
pharmaceutical carriers are only exemplary and one of
skill in the art will recognize that a very wide range
5   of such carriers may be employed.

The formulation of the formula X1 and XH
compounds with a pharmaceutical carrier can take many
forms. For example, the formulation may be a tablet,
capsule, powder, suppository, lozenge, syrup, emulsion,
10   liquid suspension or solution, or sterile injectable
liquid. The pharmaceutical compositions are prepared by
conventional techniques involving procedures such as
mixing, granulating and compressing, and dissolving the
ingredients. As will be appreciated from the foregoing
15   exemplary formulations, administration of the compounds
can be by any known route, including oral
administration, intramuscular and intravascular
injection.

The methods of treating a patient suffering from
20   a microbial disease, such as malaria, in accordance with
this invention comprise administering to the patient a
therapeutically effective amount of a compound according
to formula X1 or formula XH. Preferably, the patient
will be administered the compound in a formulation as
25   described above (i.e. in combination with a
pharmaceutical carrier), the formulation having a
therapeutically effective amount of the compound.

As used herein, "a therapeutically effective
amount" is preferably an amount that results in complete
30   remission of the disease. However, it will be
recognized that any improvement in the patient's
condition is clinically advantageous. Hence, "a
therapeutically effective amount" also encompasses
amounts of the administered compound that result in
35   partial remission of the disease or which slow or limit
the further progression of the disease, or which inhibit
the growth of the infectious agent or which reduce the
clinical signs and symptoms of the disease (for example,

fever and chills in a malaria infection). It is anticipated that therapeutically effective dosages which slow or limit the spread of the disease, or which inhibit the growth of the parasite will be particularly

5   suitable for combination with other anti-microbial drugs.

The compounds of the invention can be administered in a daily dosage schedule of from about 10 mg to about 10 g. One skilled in the art will

10   recognize that in determining the active amount of the anti-microbial compound to be administered, the activity of the specific compound selection, the age, weight and condition of the patient and the administration of other drugs to the patient must be considered.

15   The formula X1 and XH compounds may also be indirectly provided to patients in pro-drug formulations. For example, formula X1 and XH xanthones may be produced by co-administration of an oxidant agent with a corresponding substituted benzophenone under

20   physiological conditions. A pro-drug is thus defined herein as a compound which reacts under physiological conditions to produce a formula X1 or XH compound. Thus, the pro-drug for 4,5-DEAE-X would be 3,3'-bis-(β-diethylamino)ethoxy-2-hydroxy-benzophenone, and the pro-

25   drug for 2,3,4,5,6-pentahydroxyxanthone would be 2,3,3',4,4',5'-hexahydroxybenzophenone (exifone). The provision of the X1 and XH compounds in pro-drug form (i.e. the corresponding benzophenones) may be particularly useful where the oxidant agent which is

30   administered with the pro-drug is another anti-microbial agent. For example, the widely used anti-malarial agent primaquine is such an oxidant agent, and the combination of an XH pro-drug with primaquine is expected to be a particularly efficacious treatment for malaria.

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WHAT IS CLAIMED IS:

1. A compound according to formula X1 or formula XH.

2. A composition comprising a compound according to formula 1 in combination with a suitable pharmaceutical carrier.

3. A method of inhibiting the growth of a microbial pathogen comprising contacting the pathogen with a sufficient amount of a compound according to claim 1.

4. A compound of structure:

X - Y - Z

wherein X is a group capable of interacting with the iron atom in heme;

Y is a planar aromatic system capable of interacting with the porphyrin ring of heme through overlapping pi-pi orbitals; and

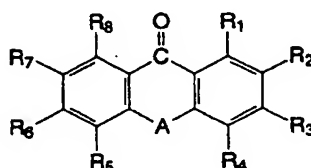Z represents one or more groups capable of interacting with at least one carboxylate side group of heme.

5. A method of inhibiting the polymerization of heme, comprising contacting heme with a compound according to claim 4.

6. A compound of claim 4 wherein the compound is of formula:

wherein:

A is oxygen, substituted antimony (stibium),
sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl,
preferably lower alkyl or lower haloalkyl wherein
"lower" means 10 or fewer carbon atoms, aryl or
5   haloaryl;

R_1-R_3 and R_6-R_8 are independently selected from
the group consisting of H, OH, halogen, aryl, arylamine,
alkyl, substituted alkyl (such as alkoxy, alkylamine,
alkylthio and haloakyl), amino, ester and nitro groups
10 and O-linked and C-linked carbohydrates;

at least one of the R_4 and R_5 substituent pair is
selected from the group consisting of amino, substituted
amino, alkylamino, substituted alkyl amino, arylamino,
amidinium, alkylamidinium, guanidinium,
15 alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy,
alkoxyamine, alkoxy-substituted amine, azido, carboxylic
esters of hydroxy, alkylhydroxy and alkoxyhydroxy
groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-$CONH_2$; and

the other member of the R_4 and R_5 substituent
20 pair is selected from the group consisting of H, OH,
halogen, aryl, arylamine, alkyl, substituted alkyl (such
as alkoxy, alkylamine, alkylthio and haloakyl), amino,
substituted amino, ester and nitro groups, O-linked and
C-linked carbohydrates, alkylamino, substituted alkyl
25 amino, arylamino, amidinium, alkylamidinium,
guanidinium, alkylguanidinium, alkylhydroxy,
alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine,
azido, carboxylic esters of hydroxy, alkylhydroxy and
alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-
30 $CONH_2$.

7.   A compound according to claim 6 wherein the
compound is selected from the group consisting of: 4,5-
dihydroxyxanthone, 2,3,4-trihydroxyxanthone, 3,4,5,6-
35 tetrahydroxyxanthone, 2,3,4,5,6-pentahydroxyxanthone,
1,3,5,6,7-pentahydroxyxanthone, 2,3,4,5,6,7-
hexahydroxyxanthone; and 4,5-bis-((β-
diethylamino)ethoxy)xanthone.

8.   A compound according to claim 6 wherein both
R_4 and R_5 are selected from the group consisting of
amino, substituted amino, alkylamino, substituted alkyl
amino, arylamino, amidinium, alkylamidinium,
guanidinium, alkylguanidinium, hydroxy, alkylhydroxy,
alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine,
azido, carboxylic esters of hydroxy, alkylhydroxy and
alkoxyhydroxy groups, COOH, alkyl-COOH, $CONH_2$ and alkyl-
$CONH_2$.

9.   A composition for the treatment of a
microbial disease consisting essentially of:
     a therapeutically effective amount of a compound
having a formula X1:
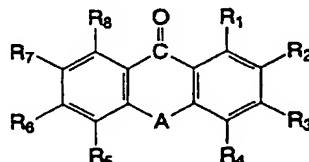
wherein A is oxygen, substituted antimony (stibium),
sulfur or N-R' wherein R' is H, hydroxy, alkyl,
haloalkyl, aryl or haloaryl, and R_1-R_8 are independently
selected from the group consisting of H, OH, aryl,
arylamine, alkyl, substituted alkyl, halogen, amino,
ester and nitro groups and O-linked and C-linked
carbohydrates; and
     a suitable pharmaceutical carrier.

10.   A method of inhibiting the growth of a
microbial pathogen, the method comprising the steps of:
     providing a composition according to claim 9;
and
     contacting the microbial pathogen with the
compound.

11.   The method of claim 10 wherein the compound
is selected from the group consisting of 2,3,4,5,6-

pentahydroxyxanthone; 2,3,4,5,6-pentaacetoxyxanthone; 2,3,4,5,6,7-hexahydroxyxanthone; 2,3,4,5,6,7-hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone; 2,3,4,5,6-pentaacetoxyacridone; 2,3,4,5,6,7-hexahydroxyacridone; 2,3,4,5,6,7-hexaacetoxyacridone; 2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6-pentaacetoxythioxanthone; 2,3,4,5,6,7-hexahydroxythioxanthone; and 2,3,4,5,6,7-hexaacetoxythioxanthone.

12. The method of claim 10 wherein the microbial pathogen is a protozoan parasite.

13. The method of claim 12 wherein the protozoan parasite is a *Plasmodium* species or a *Leishmania* species.

14. The method of claim 10 wherein the microbial pathogen is a bacterial pathogen.

15. The method of claim 10 wherein the microbial pathogen is a fungal species.

16. The method of claim 10 wherein the pathogen is a Helminth, such as a *Schistosoma* spp.

17. A method of inhibiting the growth of a protozoan parasite, the method comprising the steps of:
    providing a therapeutically effective amount of a composition comprising a compound having a formula
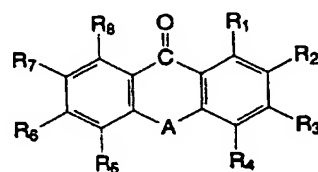
wherein A is oxygen, substituted antimony (stibium), sulfur, or N-R' wherein R' is H, hydroxy, alkyl,

haloalkyl, aryl or haloaryl, and R_1-R_8 are independently selected from the group consisting of H, OH, aryl, arylamine, alkyl, substituted alkyl, halogen, amino, ester and nitro groups, and O-linked and C-linked

5   carbohydrates; and

contacting the protozoan parasite with the compound.

18.   The method of claim 17 wherein in the

10   formula R_1 and R_8 are H.

19.   The method of claim 17 wherein the step of contacting the parasite with the compound comprises administering a therapeutically effective dosage of the

15   compound to a patient infected with the parasite.

20.   The method of claim 17 wherein the protozoan parasite is a *Plasmodium* sp. or a *Leishmania* sp.

20

21.   A method of inhibiting the growth of a protozoan parasite, the method comprising contacting the parasite with an amount of a compound selected from the following group, the amount sufficient to inhibit the

25   growth of the parasite: 2,3,4,5,6-pentahydroxyxanthone; 2,3,4,5,6-pentaacetoxyxanthone; 2,3,4,5,6,7-hexahydroxyxanthone; 2,3,4,5,6,7-hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone; 2,3,4,5,6-pentaacetoxyacridone; 2,3,4,5,6,7-hexahydroxyacridone;

30   2,3,4,5,6,7-hexaacetoxyacridone; 2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6-pentaacetoxythioxanthone; 2,3,4,5,6,7-hexahydroxythioxanthone; 2,3,4,5,6,7-hexaacetoxythioxanthone; and 4,5-bis-(diethylamino-

35   ethoxy)-xanthone.

22.   A compound of formula:
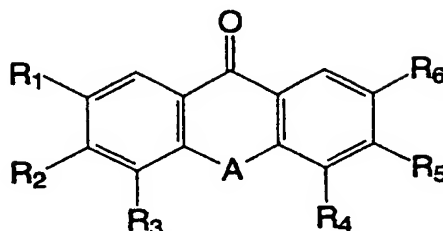
wherein A is

O, S, substituted Sb or N-R' wherein R' is H, OH, alkyl, haloalkyl, aryl or haloaryl; and

R_1-R_6 are selected from the following options:

(1) R_6 is H and R_2 - R_5 are independently selected from the group consisting of OH, halogen, $OCOCH_3$ and $OCO(CH_2)_nCH_3$ wherein n is 1-10; and

(2) R_1 - R_6 are independently selected from the group consisting of OH, halogen, $OCOCH_3$ and $OCO(CH_2)_nCH_3$ wherein n is 1-5.

23.   The compound of claim 22 wherein the compound is selected from the group consisting of: 2,3,4,5,6-pentahydroxyxanthone; 2,3,4,5,6-pentaacetoxyxanthone; 2,3,4,5,6,7-hexahydroxyxanthone; 2,3,4,5,6,7-hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone; 2,3,4,5,6-pentaacetoxyacridone; 2,3,4,5,6,7-hexahydroxyacridone; 2,3,4,5,6,7-hexaacetoxyacridone; 2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6-pentaacetoxythioxanthone; 2,3,4,5,6,7-hexahydroxythioxanthone; and 2,3,4,5,6,7-hexaacetoxythioxanthone.

24.   A therapeutic composition for inhibiting the growth of a microbial pathogen, the composition comprising:

an amount of a compound according to claim 22 sufficient to inhibit the growth of the microbial pathogen; and

a suitable pharmaceutical carrier.

25. A composition for the treatment of malaria comprising an oxidant agent and a formula XH compound pro-drug.

5   26. The composition of claim 25 wherein the oxidant agent is primaquine.

27. The composition of claim 25 wherein the pro-drug is 3,3'-diethyl-aminoethoxy-2-hydroxy-

10   benzophenone or 2,3,3',4,4',5'-hexahydroxybenzophenone.

28. A method of treating malaria comprising administering to a patient a therapeutically effective amount of primaquine in combination with a formula XH

15   compound pro-drug.

29. The method of claim 28 wherein the pro-drug is 3,3'-diethyl-aminoethoxy-2-hydroxy-benzophenone.
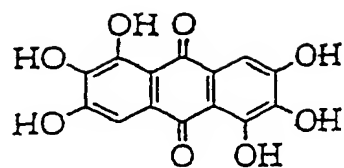
Rufigallol

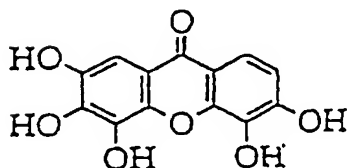*Futile redox cycling generates oxygen radicals*
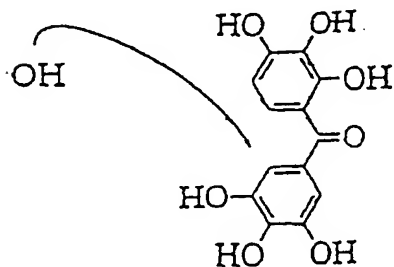
Exifone

Hydroxyl radicals attack exifone
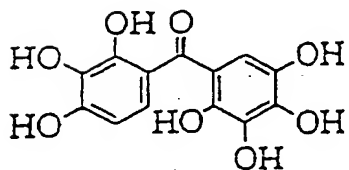
Putative intermediate: 2,3,4,5,2',3',4'-heptahydroxy-benzophenone

Cyclodehydration/cyclo-activation event

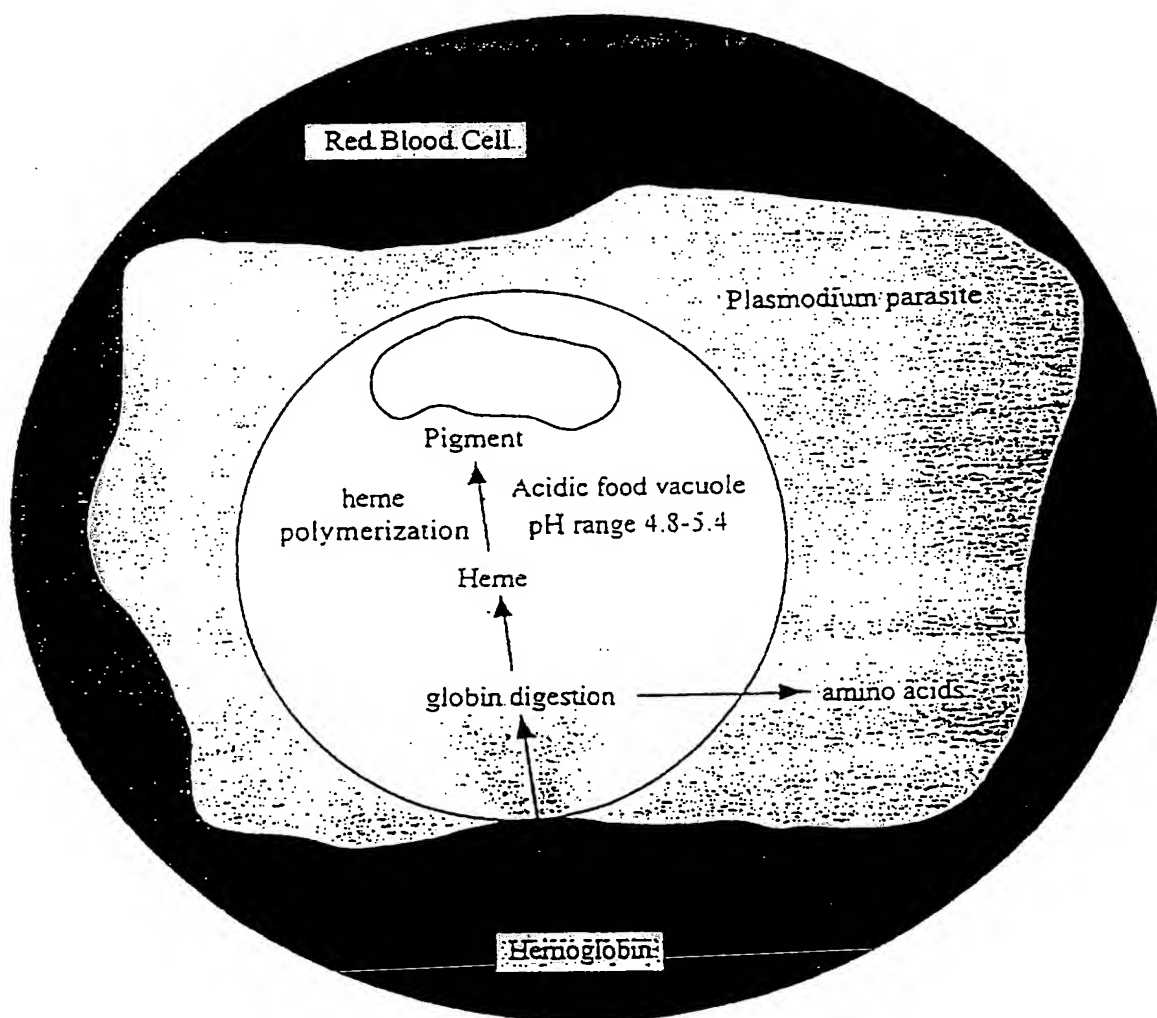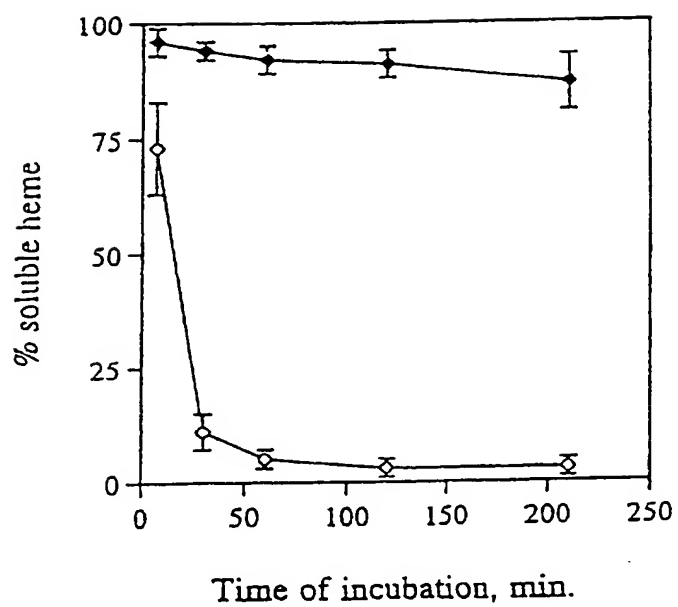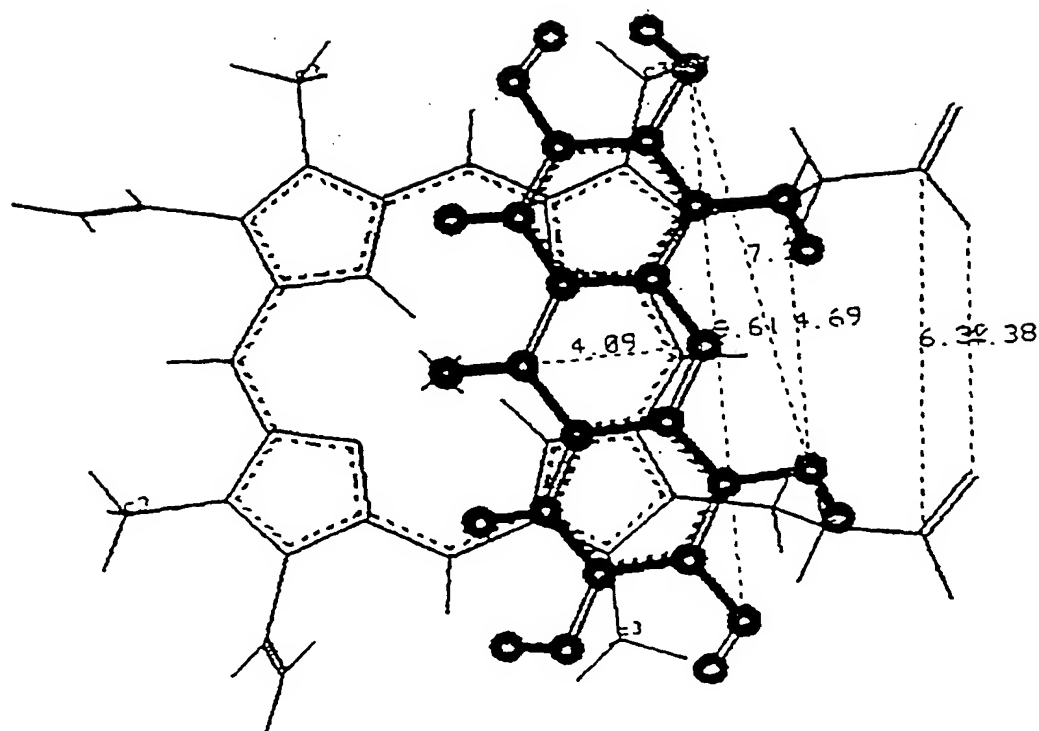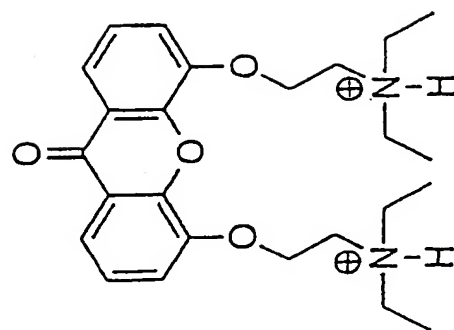Potent antimalarial agent? 2,3,4,5,6-pentahydroxy-xanthone

FIGURE 1

FIGURE 2

Time of incubation, min.

FIGURE 3

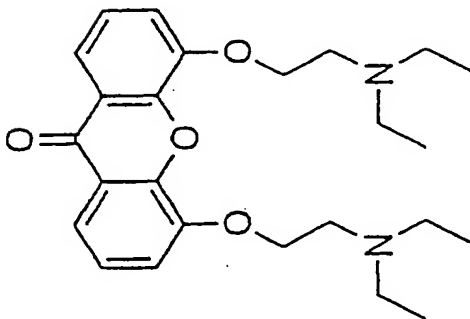# FIGURE 4

Entry into acidic digestive
vacuole (pH 4.8 to 5.5)

FIGURE 5

International application No.

PCT/US97/04965

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A01N 43/00; A61K 31/33, 31/335; C07D 311/00, 413/00, 413/14
US CL   :514/183, 455; 544/150; 549/383

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   514/183, 455; 544/150; 549/383

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, REGISTRY, CA, CAOLD, MARPAT

search terms: xanthone, parasit?, fung?, hydroxyxanthone

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAMBLOCH, H. et al. QSAR with the tuberculostatic activity of polyhydroxy xanthones and their 13C-NMR chemical shifts. Eur. J. Med. Chem. January 1985, Vol.20, No.1, pages 71-77, especially pages 73-74.	1-2, 4, 6-9, 22-24
X	US 3,887,574 a (ELLIS et al) 03 June 1975 (03/06/75), see entire document, especially column 1-3.	1-2, 4, 6-9, 22-24
X	US 5,281,620 A (DENNY et al) 25 January 1994 (25/01/94), see entire document, especially columns 1-2 and 18.	1-2, 4, 6-9, 22-24

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

°	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 JUNE 1997	0 1 AUG 1997

Name and mailing address of the ISA/US	Authorized Officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MICHAEL D. PAK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04965

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-2, 4, 6-9, and 22-24 with species A where A=oxygen

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)☆